

Approaches for Understanding the Mechanisms of Long Noncoding RNA Regulation of Gene Expression

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SUMMARY

Mammalian genomes encode tens of thousands of long noncoding RNAs (lncRNAs) that have been implicated in a diverse array of biological processes and human diseases. In recent years, the development of new tools for studying lncRNAs has enabled important progress in defining the mechanisms by which Xist and other lncRNAs function. This collective work provides a framework for how to define the mechanisms by which lncRNAs act. This includes defining lncRNA function, identifying and characterizing lncRNA–protein interactions, and lncRNA localization in the cell. In this review, we discuss various experimental approaches for deciphering lncRNA mechanisms and discuss issues and limitations in interpreting these results. We explore what these data can reveal about lncRNA function and mechanism as well as emerging insights into lncRNA biology that have been derived from these studies.

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1 INTRODUCTION

Over the last decade, tens of thousands of long noncoding RNAs (lncRNAs) have been discovered in mammalian genomes, with their numbers likely to eclipse the number of proteins (Guttman et al. 2009; Derrien et al. 2012). lncRNAs generally have similar properties to messenger RNAs in that they are often spliced, 5'-capped, and polyadenylated (Cabili et al. 2011; Derrien et al. 2012), yet are not translated into a protein product and instead function as an RNA molecule (Guttman et al. 2013). lncRNAs range in size from hundreds to tens of thousands of nucleotides in length, are expressed across a wide range of levels (Cabili et al. 2011; Derrien et al. 2012), and can localize to target sites within the nucleus or cytoplasm of the cell (Khalil et al. 2009; Cabili et al. 2015; Werner and Ruthenburg 2015). lncRNAs have begun to take a center stage in many aspects of biology, having been shown to play essential roles in diverse biological processes from development to immune responses (Rinn and Chang 2012; Sauvageau et al. 2013), and hundreds of lncRNAs are amplified, deleted, or otherwise disrupted in various human cancers and diseases (Wapinski and Chang 2011). Yet, despite the increasing prominence of lncRNAs in biology and disease, we still know very little about the mechanisms by which most lncRNAs act (Rinn and Chang 2012; Engreitz et al. 2016b), largely because they remain difficult to study.

The best-studied lncRNA is Xist, which orchestrates X-chromosome inactivation (XCI), an essential developmental process that ensures proper expression balance of genes on the X chromosome in female mammals (da Rocha and Heard 2017). Xist localizes across the entire X chromosome (Clemson et al. 1996; Engreitz et al. 2013), recruits repressive chromatin proteins to these sites (Plath et al. 2003; McHugh et al. 2015), triggers chromosome-wide transcriptional silencing (Penny et al. 1996; Wutz et al. 2002; McHugh et al. 2015), and mediates a cascade of chromosome structure changes and chromatin modifications to maintain this silenced state (da Rocha and Heard 2017).

Deciphering the mechanism by which Xist orchestrates XCI by modulating chromatin structure and silencing gene expression required the work of many laboratories and nearly 25 years of effort (da Rocha and Heard 2017). In addition to chromatin regulation, we now know that lncRNAs can play other roles in posttranscriptional gene regulation. For example, Malat1 localizes with nascent precursor messenger RNA (pre-mRNA) (Engreitz et al. 2014; West et al. 2014) around nuclear speckles (Spector and Lamond 2011), interacts with various splicing and mRNA processing proteins, and may act to control alternative splicing (Tripathi et al. 2010). Neat1 can act to nucleate

the establishment of a nuclear territory called the paraspeckle (Clemson et al. 2009), which contains various mRNA species of unknown identity (Fox et al. 2018). In the cytoplasm, NORAD can interact with mRNA translation proteins (e.g., Pumilio) and may act to titrate these proteins away from specific mRNA targets to regulate mRNA translation (Lee et al. 2016). These examples highlight the diverse roles that lncRNAs can play by interacting with different molecules in different cellular locations (Kopp and Mendell 2018).

In recent years, the development of new tools for studying lncRNAs has enabled important progress and has begun to uncover key aspects of the mechanisms by which Xist and other lncRNAs work (Engreitz et al. 2016b; Kopp and Mendell 2018). This collective work provides a framework for how to define lncRNA function and mechanism. Specifically, deciphering these mechanisms required (1) determining that the lncRNA acts as a functional RNA molecule, (2) comprehensively defining the proteins that interact with the lncRNA, (3) defining where the lncRNA binds (to genomic DNA or RNA) and in what precise location within the cell, and (4) determining how these RNA–protein components are integrated to control cellular function.

In this review, we will explore how information about lncRNA protein binding, genomic and cellular localization, and function can be integrated to understand the mechanisms of lncRNAs in gene regulation, and we will discuss the methods used for exploring these questions.

2 METHODS FOR DEFINING lncRNA FUNCTION

Understanding how any lncRNA controls cellular function requires direct perturbation followed by observations of the functional consequences in vivo (e.g., cells, tissues, or animal models). Yet, this remains challenging in practice—more so than disrupting protein-coding genes. Whereas protein-coding genes can be disrupted through small frame-shift mutations in the coding sequence, we do not know a priori which parts of a lncRNA sequence are necessary for its function and therefore whether short insertions/deletions should disrupt its function. This necessitates larger deletions, such as deletion of the entire genomic locus, to confidently disrupt the lncRNA function. Indeed, this was precisely how Xist was initially characterized. Shortly after Xist was identified as a transcript expressed from the inactive X chromosome (Xi) but not the active X (Brockdorff et al. 1991; Brown et al. 1991), it was shown that cells containing a deletion of the Xist locus failed to initiate XCI (Penny et al. 1996).

However, large deletions frequently have unintended consequences, such as by removing DNA regulatory ele-





ments contained within the lncRNA locus that can affect nearby genes (Groff et al. 2016; Engreitz et al. 2016a). These considerations must be taken into account when designing deletion experiments and interpreting their results. In fact, a recent study genetically deleted 12 lncRNA loci and found that five regulate the expression of a neighboring gene through mechanisms that do not involve the lncRNA transcript itself. Instead, several of these lncRNA promoters acted at the DNA level as *cis*-regulatory elements (enhancers) that controlled another neighboring gene, whereas others controlled expression of neighboring genes in a manner that was dependent on the active transcription—but not the sequence—of the lncRNA gene (Engreitz et al. 2016a).

Accordingly, orthogonal approaches are required to eliminate lncRNA expression to show whether a genetic locus functions through an encoded lncRNA (Engreitz et al. 2016a). In the case of Xist, additional observations were required to show that it acts as a functional RNA molecule. Specifically, (1) the Xist RNA was shown to form a unique nuclear compartment that “coats” the Xi (Clemson et al. 1996); (2) forced expression of Xist on X chromosomes or autosomes, in females or males, was shown to be sufficient to trigger gene silencing in *cis* (Wutz et al. 2002); and (3) deletion of a single region of the Xist RNA, called the A-repeat, ablates its silencing role (Wutz et al. 2002) but not its localization to Xi. Together, these sets of experiments established that the Xist RNA itself is required for initiating the process of XCI.

In this section, we will describe the various perturbation strategies for studying lncRNAs (Fig. 1) and discuss the considerations associated with interpreting each of these approaches to establish the functional role of a lncRNA *in vivo*.

2.1 Inhibition of Transcriptional Initiation

To account for the issues that arise from deleting a lncRNA locus, which can often entail deletion of thousands of nucleotides, one approach is to make a more precise deletion that simply removes the promoter region of a lncRNA transcript. This entails ablating transcription of the locus using CRISPR to delete the promoter. A similar approach is to use CRISPR interference (CRISPRi), in which a repressive protein is recruited to the promoter region of a lncRNA to block transcriptional initiation of the locus (Liu et al. 2016). Although these approaches can define whether a lncRNA-encoding locus has an effect on gene expression, it cannot distinguish between an RNA-independent (e.g., *cis*-regulatory elements or RNA transcription) and RNA-directed mechanism because CRISPRi is known to also inhibit the activity of *cis*-regulatory elements and preclude transcription (Fulco et al. 2016).

2.2 Premature Transcriptional Termination

Another approach is to retain the promoter and transcriptional initiation of the locus while eliminating the mature RNA product. One way to achieve this is through the insertion of a polyadenylation site downstream from the transcription start site, which will lead to premature termination of transcription, reduce transcription in the locus, and eliminate the mature RNA while leaving the promoter sequence intact. If a phenotype is observed in this way, one can conclude that the phenotype is not dependent on a *cis*-regulatory element but instead requires transcription activity at the locus. Yet, this approach does not fully discriminate between a sequence-dependent role for the lncRNA and the active process of transcription across the lncRNA locus.

2.3 Precise Sequence-Specific Mutagenesis and Functional Rescue

One approach to directly determine whether a lncRNA has a sequence-specific function is to delete precise sequences within the lncRNA. For example, deleting or mutating a specific protein binding site contained within a lncRNA should distinguish between sequence-independent effects of lncRNA transcription and sequence-specific effects of the lncRNA itself. This approach also has the benefit of allowing specific functions to be assigned to the mutated domains. Because this approach is often associated with identification of the proteins that interact with a specific region of a lncRNA, such perturbations can be directly complemented by functional rescue experiments that establish a direct relationship between the function of a sequence specific domain and RNA–protein function. This can be achieved by expressing a mutant RNA containing an RNA aptamer sequence (e.g., MS2 or BoxB) that can interact with the protein of interest through an engineered protein tag (e.g., MCP or λ N) (Keryer-Bibens et al. 2008). Although this approach was critical for defining the functions of several Xist domains (Chen et al. 2016; Pintacuda et al. 2017), it generally requires knowledge of the protein binding sites on a lncRNA.

2.4 Posttranscriptional Degradation

A more direct way to determine whether a lncRNA acts directly as an RNA molecule is to degrade the mature RNA after it is fully transcribed. There are several approaches for doing this, including antisense DNA oligonucleotides (ASOs) that anneal to the lncRNA and trigger RNase H-mediated degradation (Liang et al. 2017) and RNA interference (RNAi) in which an antisense RNA anneals to

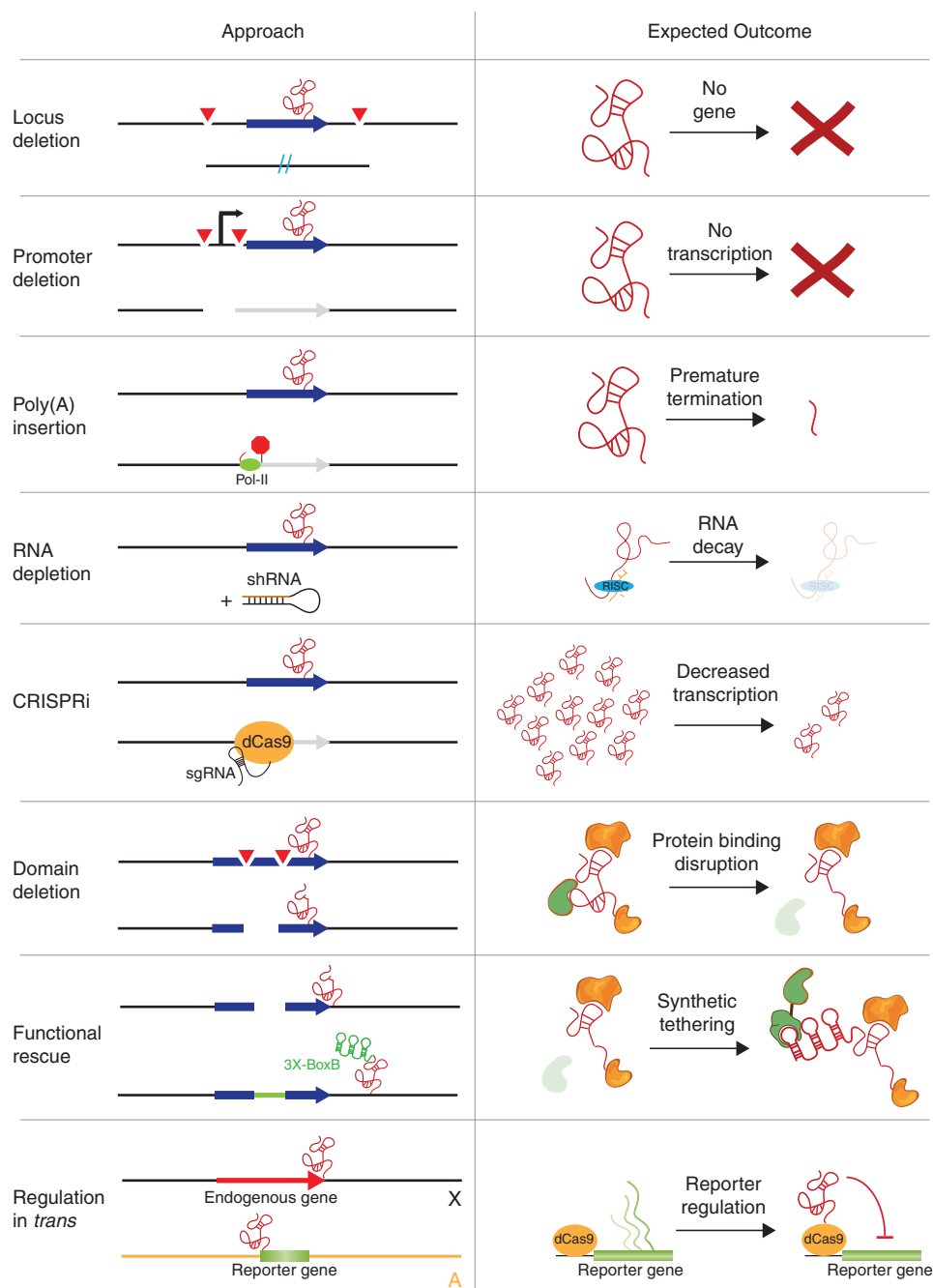


Figure 1. Genetic strategies to study long noncoding RNA (lncRNA) function. Locus and promoter deletions (e.g., by CRISPR [red arrows]) can generate null alleles such that the gene that encodes the lncRNA is removed from the genome or the promoter is removed preventing lncRNA transcription. However, both of these approaches could also delete functional regulatory DNA sequences contained within these DNA sequences. Inserting a poly(A) transcription termination signal does not disrupt transcription initiation at the lncRNA locus but terminates transcription prematurely. Antisense oligonucleotides or RNA interference (RNAi) can degrade the lncRNA posttranscriptionally, but there may be off-target effects that are often hard to predict. Recruitment of dCas9 (usually fused to a repressor domain such as KRAB) by short guide RNAs using CRISPR leads to decreased transcription of the lncRNA without mutating the DNA sequence, but because it also precludes recruitment of PolII to enhancer elements, it can also impact functions mediated through DNA elements. Deleting a specific lncRNA–protein interaction domain can impact protein binding and can be complemented with synthetic rescue experiments that tether the protein to the mutant RNA. Expressing a *cis*-acting lncRNA from a different locus and chromatin context can reveal mechanisms of action and gene regulatory function in *trans*.



the lncRNA and triggers argonaute-mediated degradation (Ghildiyal and Zamore 2009). Although these represent important methods, in practice they are often less precise than genetic engineering because hybridization of these short oligonucleotide sequences can have off-target effects that are difficult to identify. Indeed, several lncRNAs that were initially characterized using RNAi, ASOs, or similar methods, have recently been reevaluated by genetic studies and shown to have distinct functions from those previously reported. These include several examples that have since been shown to act as *cis*-regulatory elements rather than functional RNA molecules (Engreitz et al. 2016a; Groff et al. 2016). For example, lincRNA-P21 was initially described as acting in *trans* based on functions identified by RNAi-mediated depletion of the lncRNA (Huarte et al. 2010). However, subsequent perturbation of this lncRNA using genetic dissection suggested that the RNA product may be dispensable and the observed phenotype may simply be explained by the *cis*-regulatory function of the DNA element (Groff et al. 2016). Indeed, several lncRNAs previously defined to contain functional roles based on RNAi in embryonic stem cells (ESCs) (Guttman et al. 2011) have been shown to have functions that occur in *cis*, independent of their RNA products (Engreitz et al. 2016a). Although these results do not exclude the possibility that these RNA products may still have other functional roles, they do highlight the importance of orthogonal approaches and validation to directly confirm the functional role of a lncRNA.

Although the individual approaches above have caveats regarding their interpretation, when performed in combinations they can provide high confidence about the function of a lncRNA. One orthogonal approach to gain additional confidence is to complement genetic perturbation with a rescue construct in *trans*. This may often fail, however, because many lncRNA functions are associated with 3D proximity and therefore simply expressing the lncRNA from an exogenous site may fail to rescue the defect. Other approaches, such as targeted recruitment of a lncRNA to a genomic DNA locus, or rescue of individual RNA-protein interactions, can overcome this issue, but requires more precise knowledge of the mechanisms by which a lncRNA may work. Recently, several new technologies have been developed that enable the direct assessment of the functional role of a lncRNA through targeted recruitment of the lncRNA in *trans* to a reporter gene. These approaches have been used for studying HOTAIR by recruitment through the GAL4-UAS system (Portoso et al. 2017). More recently, a method called CRISPR-Display has generalized this approach in a manner that enables programmable recruitment of lncRNAs to different DNA regions on larger scales (Shechner et al. 2015).

Because these approaches recruit a lncRNA independently of its transcriptional and local environment, presence of a function in these assays indicates a role for the RNA transcript itself. However, the absence of a phenotype in such an assay does not preclude a critical role for the lncRNA in its endogenous chromatin environment, which may be distinct from the chromatin context that is present at the reporter gene.

3 DEFINING lncRNA-PROTEIN INTERACTIONS AND THEIR FUNCTIONAL CONTRIBUTIONS

Determining how Xist mediates transcriptional silencing required advances in biochemical purification approaches and mass spectrometry to comprehensively define the proteins that directly interact with Xist (Chu et al. 2015; McHugh et al. 2015). These studies identified numerous proteins that begin to explain key aspects of how Xist works to orchestrate the process of XCI. Mapping in which these proteins bind across the Xist lncRNA showed that they bind at discrete locations (Chen et al. 2016; Chu et al. 2015; Cirillo et al. 2016; Pintacuda et al. 2017) and enabled genetic studies to delete individual domains to study the functional contribution of each lncRNA-protein complex in orchestrating XCI. Together, these studies uncovered the Xist-protein interactions that are required for transcriptional silencing, recruitment of various chromatin regulators (e.g., PRC1 and PRC2), localization to genomic DNA, and chromosome-wide spreading.

In this section, we will discuss methods for defining (1) which proteins interact with a lncRNA, (2) where on the RNA each of these proteins binds, and (3) the functional role of each lncRNA-protein interaction. We will highlight important issues and consideration that are needed for designing and interpreting lncRNA-protein interactions.

3.1 Methods for Comprehensively Defining Proteins That Bind to a Specific lncRNA

There are several methods for purifying a specific lncRNA and defining associated proteins. These methods differ in how the RNA is purified after incubation with proteins in solution or after cross-linking of RNA-protein complexes *in vivo*.

One general approach for purifying RNA is to attach an RNA sequence that forms a tight interaction with a defined protein complex. For example, a hairpin structure from the MS2 RNA forms a tight interaction with the bacteriophage MS2 viral coat protein (Keryer-Bibens et al. 2008). In this approach, repeats of the MS2-binding RNA are attached to an RNA of interest and the RNA is captured by immobilizing the associated RNA onto a protein-bound

resin. There are many variants of this approach using different combinations of RNA–protein substrates that have various affinities and therefore enable different stringencies (McHugh et al. 2014). In addition, there are variants such as the S1 RNA aptamer that mimics biotin in that it can interact directly with streptavidin. Because it has a lower affinity than biotin, it can be selectively eluted through the addition of free biotin (Srisawat and Engelke 2002). This specific elution strategy provides important benefits for reducing the background, and other similar approaches have been developed for this reason. For example, the Csy4 protein, derived from the bacterial CRISPR system, binds tightly to its cognate RNA, more tightly than MS2 or PP7 coat protein interactions, while enabling specific elution through the addition of imidazole (Lee et al. 2013).

The other general approach for RNA purification is based on hybridization of antisense oligonucleotides (Chu et al. 2011; Engreitz et al. 2013; Simon et al. 2013; Rogell et al. 2017). These approaches generally use biotin labeled oligonucleotides to hybridize to a specific RNA sequence and capture it, and its associated proteins, from a complex mixture. These strategies vary in several ways: (1) some use individual oligonucleotides (Simon et al. 2013; Rogell et al. 2017) whereas others use pools of oligonucleotides that tile across the RNA of interest (Chu et al. 2011; Engreitz et al. 2013); (2) some methods use short oligonucleotides (Chu et al. 2011; Simon et al. 2013; Rogell et al. 2017) and others use long oligonucleotides (Engreitz et al. 2013); and (3) some use unmodified nucleotides (Chu et al. 2011; Engreitz et al. 2013; Simon et al. 2013), whereas others use locked nucleic acids (Rogell et al. 2017). The key consideration in choosing between these parameters comes down to specificity of the hybrid and the stringency of the purification conditions used in the purification procedure.

Until recently, these RNA purification approaches were generally performed after incubating a labeled RNA with protein obtained from total cell extracts. Although such an approach had proven successful for defining proteins that interact with classical noncoding RNAs (ncRNAs) (such as snRNPs and 7SK), they proved more problematic for studying lncRNAs. For example, early attempts to purify Xist and its associated proteins used an affinity tag coupled to the RNA (Brown and Baldry 1996) to identify an interaction between Xist and heterogeneous nuclear ribonucleoprotein C (hnRNPC); yet, this interaction was insufficient to explain the silencing role mediated by Xist. Similar approaches led to the detection of specific proteins that interact with several lncRNAs (e.g., NORAD, linc-P21) (Huarte et al. 2010; Lee et al. 2016), but they are usually limited to identifying abundant RNA binding proteins (e.g., Pumilio, heterogeneous nuclear ribonucleoprotein K [hnRNPK])

and do not provide a comprehensive picture of the proteins that interact with a lncRNA.

These results highlight one of the key challenges with RNA purification methods that are performed in solution: Many biologically relevant RNA-binding proteins (RBPs) that interact with a lncRNA are not identified. The issue is that when incubated in solution, abundant RBPs can outcompete lower abundance proteins. Thus, it is easier to observe interactions with abundant RBPs, but not necessarily other bona fide RBPs that interact with the lncRNA in cells, which may be present at much lower concentrations. This is true even if specific RBPs have significantly higher affinity because the differences in concentration are often large enough to overcome differences in affinity. Moreover, because these approaches break open the cell, such that the concentrations of proteins are uniform throughout solution, the results may not accurately reflect the actual local concentration of protein complexes near an lncRNA in vivo (Darnell 2010; Mili and Steitz 2004; McHugh et al. 2014). Consequently, promiscuous RNA–protein interactions might form in vitro that do not occur in vivo, because of compartmentalization, sequestration, and phase separation. Accordingly, despite numerous attempts to define the mechanism of how Xist silences transcription, until recently it remained unclear what RBPs interact with Xist and what components are required for Xist-mediated transcriptional silencing.

To address this challenge, several groups developed RNA purification methods (e.g., ChIRP-MS, CHART-MS, and RAP-MS) (Fig. 2) that can both comprehensively define the proteins that interact with a specific lncRNA and at the same time achieve the specificity required to only identify interactions that occur in vivo (West et al. 2014; Chu et al. 2015; McHugh et al. 2015). These methods achieve this goal by using in vivo cross-linking to fix RNA–protein interactions that occur inside cells and then purifying the RNA of interest using extremely stringent, even denaturing, conditions to remove background associating proteins. These methods differ in the type of cross-linking that is used to fix RNA–protein interactions, which impacts the types of proteins that are identified by each method. For example, RAP uses ultraviolet (UV) cross-linking to fix only those proteins that directly interact with an RNA, whereas ChIRP uses formaldehyde cross-linking which also cross-links interacting proteins. After purification, proteins that are covalently cross-linked to a given RNA are identified using mass spectrometry.

These approaches led to the discovery of dozens of proteins that interact with Xist. Although there are differences between the proteins identified by these approaches, the proteins identified using UV cross-linking would be expected to represent proteins that directly interact with

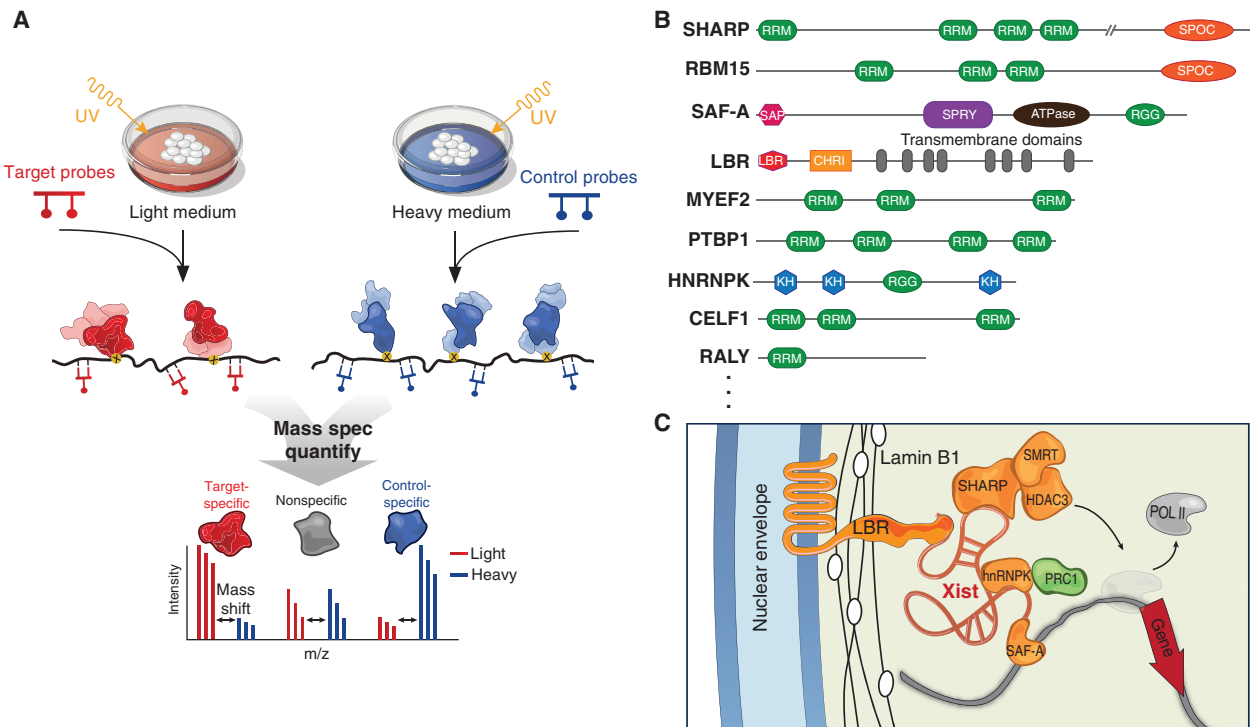


Figure 2. Comprehensive identification of proteins that interact with long noncoding RNAs (lncRNAs). (A) Anti-sense capture probes are hybridized to the target lncRNA in cross-linked lysate that has been SILAC-labeled with heavy or light amino acids. Following capture and stringent washes, the purified proteins are identified using mass spectrometry. Nonspecific background peptides found in the control sample are subtracted out, identifying specific interacting proteins. (B) Example list of Xist-interacting proteins found by multiple Xist purification and mass spectrometry (MS) methods, and their associated protein domains. (C) Current model for how Xist interacts directly and indirectly with proteins in the cell to silence transcription on Xi, revealed by recent MS studies. Xist RNA associates with genomic DNA through a direct interaction with SAF-A/hnRNPU, and binds SHARP/SPEN to recruit SMRT and HDAC3 to deacetylate histone H3 and exclude RNA polymerase II from nearby loci. Xist also binds hnRNP-K which recruits the PRC1 complex, leading to histone ubiquitination and subsequent recruitment of the PRC2 complex and its H3K27me3 histone methyltransferase activity. Lamin B receptor (LBR) binding of Xist recruits Xi to the nuclear lamina, creating a repressive nuclear territory and enabling further spreading by Xist. Proteins depicted as directly touching Xist RNA in this figure were identified by MS studies as directly bound to Xist; the other proteins depicted were subsequently identified by orthogonal studies. (Adapted, with permission, from McHugh et al. 2015.)

an RNA, whereas those identified by formaldehyde would also include proteins that associate with an RNA via protein–protein interactions. Importantly, the core protein components identified are similar between both methods (Fig. 2) (Chu et al. 2015; McHugh et al. 2015).

Cross-linking studies identified the proteins that explain eviction of RNA polymerase II and transcriptional silencing of the X chromosome. Specifically, numerous analyses identified SHARP (also known as SPEN) as a direct Xist-interacting protein that is required for chromosome-wide transcriptional silencing on the X chromosome (Chu et al. 2015; McHugh et al. 2015; Moindrot et al. 2015; Monfort et al. 2015). Determination of the extended protein complex interacting with SHARP revealed an essential role for SMRT and HDAC3 in the exclusion of RNA polymerase

II from the X-chromosome territory and subsequent transcriptional silencing (McHugh et al. 2015). These results indicated that Xist initiates transcriptional silencing by directly binding to SHARP, recruiting SMRT and HDAC3 to trigger deacetylation on the X chromosome. Additionally, these studies identified hnRNP-K as a direct Xist-interacting protein (Chu et al. 2015; Pintacuda et al. 2017). Analysis of the extended interaction network showed that hnRNP-K acts to recruit the PRC1 complex to the X chromosome through the noncanonical PCGF3/5 complex (Pintacuda et al. 2017). This leads to ubiquitylation of chromatin and subsequent recruitment of PRC2 and its associated H3K27me3 modifications across the X chromosome (Almeida et al. 2017), thus reconciling prior observations that PRC2 is recruited to Xi but is not recruited through a direct

interaction between PRC2 and the Xist RNA. These studies also identified SAF-A (also known as heterogeneous nuclear ribonucleoprotein U [hnRNP-U]) and showed that this interaction is required for tethering the Xist RNA to genomic DNA sites on the X chromosome (Hasegawa et al. 2010; Chu et al. 2015; McHugh et al. 2015). Finally, these studies identified the lamin B receptor (LBR) and showed that this interaction is required for enabling Xist to spread across the X chromosome (McHugh et al. 2015; Chen et al. 2016). Specifically, the Xist–LBR interaction is required for recruiting the X chromosome to the nuclear lamina and by doing so leads to three-dimensional (3D) structural changes on the X chromosome that allow Xist to spread to additional DNA sites (Chen et al. 2016).

Importantly, the ability to comprehensively define the proteins and complexes that interact with Xist provided the first insights into the mechanisms by which Xist orchestrates XCI. Yet, despite advances in methods for purifying a lncRNA, these methods have been applied only to a relatively small number of lncRNAs because they remain technically challenging. Specifically, (1) most of these tools are limited to studying relatively highly abundant lncRNAs (i.e., Xist, Malat1) and accordingly do not work well for the vast majority of lncRNAs; (2) these methods often require large numbers of cells, limiting their usage to cell types that can be easily grown in culture; and accordingly will not work for primary cells used for studying human disease, and (3) these methods often require extensive optimization for each lncRNA and thus are not amenable to systematic characterization of many lncRNAs. Future work will be needed to adapt these approaches to enable greater sensitivity and scalability for studying a larger number of lncRNAs across broader contexts.

3.2 Mapping lncRNAs That Interact with Specific Proteins

To overcome the limitation in detection sensitivity imposed by RNA-centric purifications, attempts were made to use information about XCI to guide identification of the proteins that might be involved. One important discovery was that the process of XCI is associated with recruitment of the PRC2 complex and its associated H3K27me3 marks across the X chromosome (Plath et al. 2003). Based on this observation, the interaction between Xist and PRC2 was tested using RNA immunoprecipitation (RIP) experiments. RIP uses native purification conditions in cross-linked or non-cross-linked cells to immunoprecipitate proteins and measure their associated RNAs (Zhao et al. 2008). Indeed, an interaction between Xist and PRC2 was shown to occur using these methods. Moreover, PRC2 was shown to interact with a truncated Xist RNA that just contained the

A-repeat region of Xist (Zhao et al. 2008), the RNA region required for transcriptional silencing (Wutz et al. 2002). These observations led to a model whereby Xist directly binds to PRC2 and recruits this repressive chromatin complex to the X chromosome to mediate transcriptional silencing (Zhao et al. 2008).

More generally, observation of an interaction between Xist and PRC2 led to intense study of the PRC2 complex as an RNA binding protein, with recent studies arguing that PRC2 binds to hundreds of different lncRNAs. This insight led to the proposal that lncRNAs act to recruit PRC2 to genomic DNA targets, among other roles (Khalil et al. 2009; Zhao et al. 2010; Guttman et al. 2011).

Although this result seemed to indicate a potential mechanism for how Xist-mediated transcriptional silencing might occur, several subsequent observations are incompatible with this model. Specifically, deletion of PRC2 components that prevent its recruitment to the X chromosome have no impact on Xist-mediated transcriptional silencing (Kalantry and Magnuson 2006; Schoeffer et al. 2006) and deletion of the A-repeat from Xist does not preclude PRC2 recruitment to the X chromosome (Plath et al. 2003; Kohlmaier et al. 2004; da Rocha et al. 2014; McHugh et al. 2015). Moreover, the distance between Xist and PRC2 on the inactive X chromosome as measured by super-resolution microscopy appears to be incompatible with direct binding (Cerase et al. 2014). In addition, recent studies purifying Xist using denaturing conditions failed to identify an interaction between Xist and any of the PRC2 components *in vivo* (Chu et al. 2015; McHugh et al. 2015; Minajigi et al. 2015). Instead, these studies identified other RBPs that directly bind to Xist, and are essential for PRC2 recruitment across the X chromosome. Furthermore, it is now clear that PRC2 is also dispensable for the functions of other lncRNAs, including HOTAIR, which it had been previously reported to bind (Portoso et al. 2017).

This discrepancy between the biochemical evidence supporting lncRNA–PRC2 interactions, and functional evidence showing that PRC2 is dispensable for Xist function, highlight important considerations for studying lncRNA–protein interactions. Specifically, interactions that are observed using immunoprecipitation methods do not necessarily reflect interactions that occur *in vivo*, but could instead reflect those that form in solution after cell lysis. Indeed, in a classic experiment, Mili and Steitz showed that immunoprecipitation methods can indeed identify RNA–protein interactions that do not occur *in vivo*, but rather form in solution after lysis (Mili and Steitz 2004). Consistent with this explanation, several recent *in vitro* experiments have shown that PRC2 components bind with high affinity to all RNAs, including bacterial RNAs



(Davidovich et al. 2013), suggesting that detecting their interactions with Xist—and possibly other lncRNAs—may represent high-affinity associations that may not be functionally required.

To date, most studies of lncRNA–protein interactions have used RIP, which has led to reports of numerous additional chromatin complexes that interact with various lncRNAs (Guttman et al. 2011). Accordingly, these same considerations must be taken into account when exploring the functional relevance of these interactions.

To account for the limited stringency afforded by the RIP method, the cross-linking and immunoprecipitation (CLIP) method was developed (Darnell 2010). CLIP uses UV cross-linking to form covalent interactions inside cells between directly interacting RNA and protein, followed by purification using more stringent wash conditions (i.e., 1 M salt) and subsequent separation through a denaturing gel and transfer to a nitrocellulose membrane to enrich for in vivo cross-linked RNA–protein interactions (Fig. 3A) (Darnell 2010). Importantly, the key difference between RIP and CLIP is the stringency of the wash conditions and denaturing gel separation used rather than the cross-linking itself. In fact, RIP can be performed in UV cross-linked cells,

but does not achieve the same stringency as CLIP without incorporating the purification of RNA–protein complexes from a denaturing gel. Because of this increased stringency, CLIP methods have emerged as the gold-standard for defining in vivo RNA–protein interactions.

Despite the critical advantages of the CLIP method, simply observing sequencing reads in a CLIP experiment does not necessarily indicate an in vivo RNA–protein interaction, and careful controls need to be designed, especially when studying proteins that may not bind RNA or that may bind to only a few RNAs in vivo.

3.3 Methods for Mapping the Precise Protein Binding Sites on RNA

CLIP also enables the identification of the direct RNA binding site of a protein—a necessary prerequisite for functional characterization of lncRNA–protein interactions. CLIP entails controlled RNase digestion of RNA into small fragments before immunoprecipitation of the protein. Accordingly, the only RNA fragment that will be purified is the region that is directly cross-linked to the protein of interest. Although this approach hones in on the region of the RNA

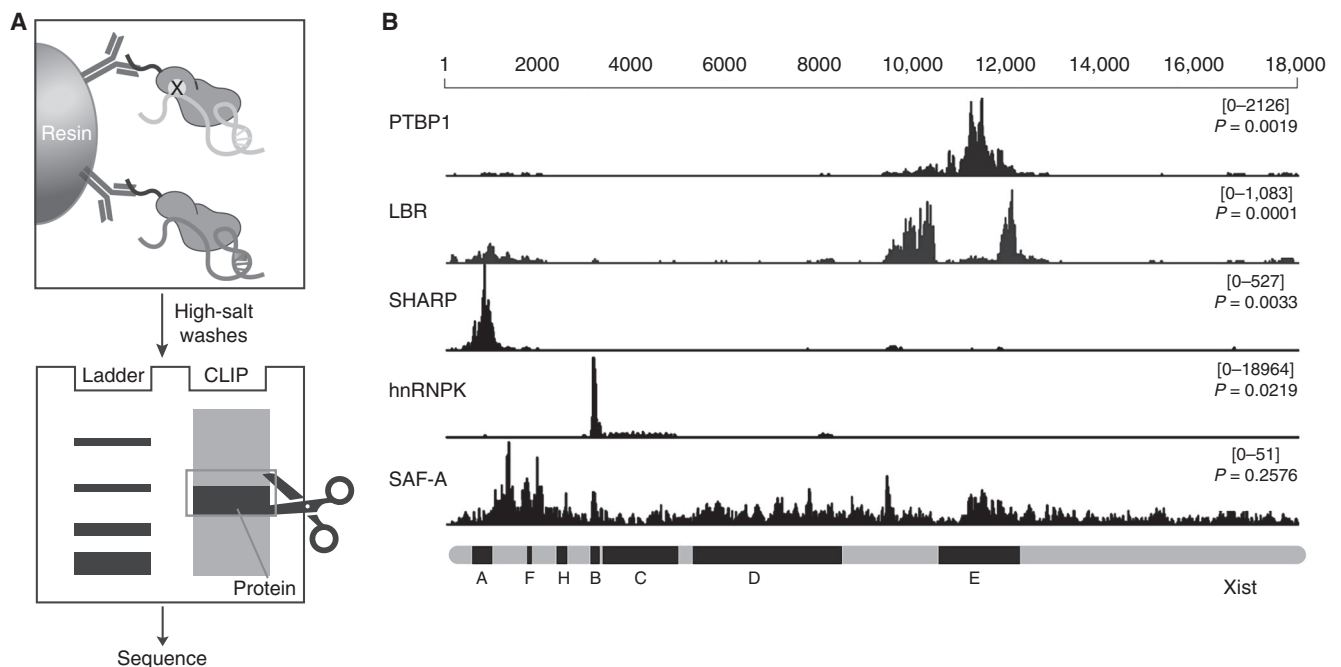


Figure 3. Cross-linking and immunoprecipitation (CLIP) methods identify protein binding sites on long noncoding RNAs (lncRNAs). (A) An overview of the CLIP method. RNA–protein interactions are cross-linked using ultraviolet (UV) light (depicted by X). Purification and stringent washes are followed by gel extraction of cross-linked RNA–protein complexes, and the purified RNA fragments are sequenced. (B) Example of protein interaction domain identification on Xist by CLIP (Cirillo et al. 2016). Although SAF-A/hnRNP-U has a broad binding distribution on Xist, other interacting proteins have clearly defined binding domains. (B, From Cirillo et al. 2016, adapted, with permission.)

that is bound by a protein, it does not resolve the precise nucleotides that are bound by a protein. To surmount this problem, several groups have developed adaptations of the CLIP method that more accurately resolve the sites of interaction. Although several such methods have been developed, one of the most popular strategies takes advantage of the fact that UV cross-linking creates an irreversible RNA–protein linkage, producing a peptide adduct present on the purified RNA after immunoprecipitation. Because reverse transcriptase often pauses at these adducts, the locations of the resulting truncated cDNA products can be mapped using sequencing to identify the likely cross-link sites (Konig et al. 2010).

CLIP has been successfully used to define the precise RNA binding sites of numerous RNA binding proteins (Darnell 2010), including those of several on Xist (Fig. 3B) (Chen et al. 2016; Cirillo et al. 2016). For example, mapping the SHARP protein by CLIP showed that it binds to the highly-conserved A-repeat domain of Xist, the domain that was previously shown to be required for transcriptional silencing (Chu et al. 2015; Chen et al. 2016). Interestingly, CLIP studies of hnRNPK showed that it interacts specifically with the B-repeat region of Xist (Cirillo et al. 2016; Pintacuda et al. 2017), a domain that was previously shown to be required for PRC2 recruitment to the X chromosome (da Rocha et al. 2014). CLIP studies of LBR identified three distinct sites on the Xist RNA, including a region overlapping the A-repeat deletion (Chen et al. 2016; Cirillo et al. 2016). Interestingly, the interaction between Xist and LBR appears to require each of the individual sites, suggesting that these regions interact to form a higher-order structured region of the RNA (Chen et al. 2016). Finally, CLIP studies of SAF-A showed that it binds to many regions across the Xist lncRNA and appears to recognize the RNA in a promiscuous manner, lacking precise sequences or structural recognition motifs (Cirillo et al. 2016). This is consistent with recent *in vitro* studies showing that the RGG domains of SAF-A bind broadly to many different RNA sequences (Ozdilek et al. 2017).

These different binding patterns can provide functional and mechanistic insights into how these RBP complexes work. For example, in the case of SAF-A, which appears to bind broadly across the Xist RNA, many interactions between Xist and SAF-A may lead to high-avidity interactions that create a tight interaction of the Xist lncRNA with chromatin.

Importantly, all of these biochemical measurements represent an average profile of interactions occurring across millions of cells and do not provide information about whether these interactions occur simultaneously with a single RNA molecule. Novel methods will be needed to determine the structures and stoichiometries of these complexes,

such information will provide critical new insights into lncRNA mechanisms.

3.4 Methods for Studying the Function of Individual lncRNA–Protein Interactions

There are several approaches to defining the function of a lncRNA–protein interaction. For example, targeted deletion of the RNA binding site that is recognized by a protein enables studies of the specific RNA–protein interaction without impacting other roles that an RNA binding protein might play. Indeed, this approach was used to study the Xist interactions with SHARP, LBR, and hnRNPK (Chen et al. 2016; Almeida et al. 2017; Pintacuda et al. 2017). However, this method only works when there is a clearly defined binding site for a protein on the lncRNA. If a protein binds broadly across an RNA, such as SAF-A on Xist, targeted mutagenesis of the RNA will not work. In such cases, deletion of the RNA binding domain of the protein can also be used to disrupt this interaction. However, in this case, the mutation will also impact the interaction between the RBP and other RNAs with which it might associate.

Importantly, in this mutagenesis approach, any observed phenotypes can be confirmed by performing rescue experiments in which the RBP and RNA are tethered together independently to determine if their interaction can explain the observed phenotype. For example, a mutant RNA (lacking a protein binding site) can be fused to the BoxB RNA aptamer and coexpressed with the protein containing a λ N peptide that will tightly bind to BoxB. In this case, if the deleted domain is critical for explaining a phenotype because of the interacting protein, then the synthetic rescue should no longer show this observed phenotype. This approach was used to show the essential role of LBR in enabling Xist spreading (Chen et al. 2016), RBM15 in recruiting YTHDC1 (Patil et al. 2016), and in hnRNPK binding and recruiting PRC1 (Almeida et al. 2017; Pintacuda et al. 2017).

4 METHODS FOR DETERMINING WHERE lncRNAs LOCALIZE

Understanding how lncRNAs work requires a detailed understanding of what the direct targets of each lncRNA are—including genomic DNA and RNA targets—and where in the cell these interactions occur. The subcellular localizations of lncRNAs provide some of the greatest clues about their biological roles and molecular functions. As functional molecules, lncRNAs must localize to their sites of action within the cell. For instance, a lncRNA that localizes to subnuclear compartments beyond its site of transcription could regulate gene expression or RNA biogenesis *in trans*,



but is unlikely to be translated by ribosomes, whereas an exclusively cytoplasmic lncRNA would not be expected to have a direct role in altering transcription or chromatin structure, but instead might be involved in regulating proteins or other RNAs after they are produced. Moreover, the subcellular localization of lncRNAs can be a consequence of interacting with proteins, or they can recruit proteins to specific sites, structures, and territories to integrate functions or sequester molecules away from their targets.

Indeed, one of the earliest and most significant insights into Xist function was the visual observation that the Xist RNA localizes to a single chromosome in female cell nuclei, apparently coating the inactive X (Brockdorff et al. 1991; Brown et al. 1991; Clemson et al. 1996). It was this observation that led to the initial proposal that Xist acts through a functional lncRNA—not its protein product—by spreading in *cis* across the chromosome from which it is expressed, to broadly silence transcription. Subsequent experiments in which Xist was integrated into autosomal regions (Lee et al. 1996; Herzing et al. 1997) showed that Xist similarly spreads across these autosomal regions, showing that the affinity of Xist RNA is not for particular DNA sequences but instead is related to the direct physical connection between the gene expressing Xist RNA and the DNA it binds. These observations established the core model for how the Xist RNA works. More recently, high-resolution mapping of Xist localization by molecular methods revealed in detail the underlying relationships of Xist spreading with 3D spatial proximity, chromatin structure, and the kinetics of gene silencing across X (Engreitz et al. 2013).

In this section, we will discuss the various methods for studying lncRNA localization within the cell (Fig. 4).

4.1 Direct Visualization of lncRNAs by Microscopy

Fluorescence microscopy has proven to be one of the most powerful tools for studying lncRNA localization, principally through fluorescence in situ hybridization (FISH) and immunofluorescence (IF). In FISH, fixed cells are hybridized with fluorescently labeled probes antisense to the RNA target and visualized by light microscopy. In fact, Xist was initially shown to localize to the Barr body at the nuclear periphery and to broadly coat the inactive X using FISH (Clemson et al. 1996). These observations directly linked the RNA molecule itself with the subnuclear territory of X inactivation. Importantly, these studies used probes that distinguished nascent Xist RNA—produced at the XIC—from the spliced, polyadenylated, mature Xist RNA that spreads over the chromosome. Subsequent FISH studies performed throughout development and in differentiating female ESCs observed the individual stages of Xist-induced silencing of X: initiation of Xist expression from the XIC

on the presumptive Xi, widespread localization of spliced Xist concomitant with propagation of inactivation in *cis*, comigration with the Xi to the nuclear periphery, and continued association with Xi throughout most of the cell cycle, even in the absence of continued Xist transcription. Immunofluorescence, wherein specific proteins in fixed cells are labeled using fluorescent antibodies, combined with Xist FISH, showed that Xist localization and spreading precedes hallmarks of transcriptional repression, such as histone deacetylation and methylation, DNA methylation, and exclusion of Pol II. These observations, when combined with genetic experiments described earlier in this review, strongly implicated the RNA itself as required for establishing X inactivation, even though the mechanistic details remained mysterious.

Improvements in microscopy and staining revealed subchromosomal enrichment of Xist RNA at gene-rich “G-light” bands on Xi, and an apparently low affinity for gene-poor heterochromatic regions (Duthie et al. 1999), with spreading into active gene regions requiring the A-repeat domain (Wutz et al. 2002), leading to the model that Xist initially localizes to high-affinity sites before spreading to the rest of the chromosome. Recently, super-resolution 3D structured illumination microscopy (3D-SIM) of the Xi confirmed that Xist RNA initially localizes to discrete foci before chromatin compaction, followed by spreading to occupy discrete chromatin domains in close association with the nuclear matrix protein SAF-A (previously shown to be required for Xist association with the Xi [Hasegawa et al. 2010]) and preceding Pol II exclusion but not involving repressive chromatin modifications, such as H3K27me3 (Cerase et al. 2014).

4.2 Methods for Genome-Wide Mapping of lncRNA Localization

Although imaging provided key insights into how Xist works, these approaches could not define where an RNA binds to genomic DNA at high resolution. Accordingly, it was still unclear how Xist spread across the X chromosome on initiation of XCI.

Recently, several methods to map lncRNA localization at high resolution using antisense purification coupled with sequencing of genomic DNA have been developed. These methods take advantage of labeled antisense probes to purify target RNAs from cross-linked cell lysates and identify DNA or RNA interactions by sequencing (Chu et al. 2011; Engreitz et al. 2013; Simon et al. 2013), an approach that is conceptually similar to the ChIP-seq method used for mapping protein–DNA interactions. Indeed, the use of different cross-linking agents, such as formaldehyde or pso-

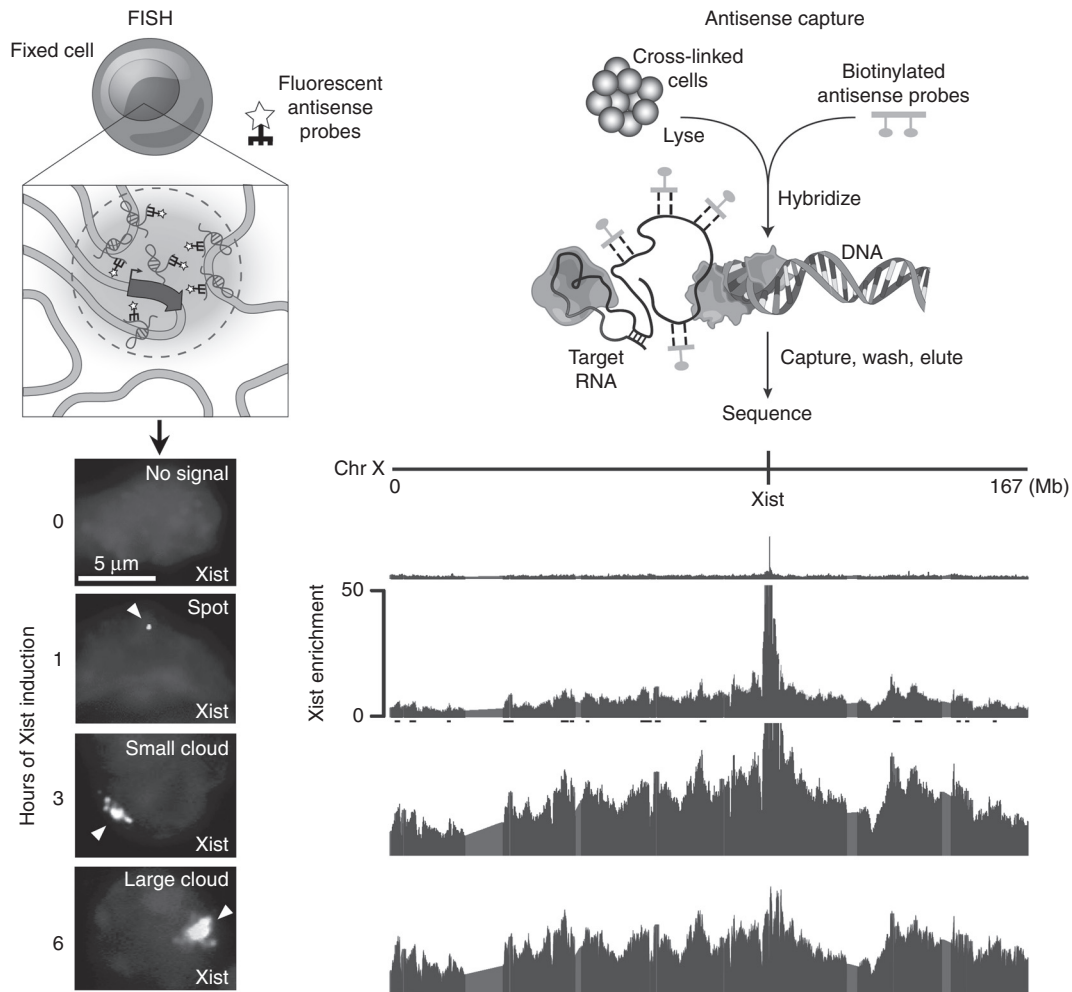


Figure 4. Methods to study long noncoding RNA (lncRNA) localization. Fluorescence in situ hybridization (FISH) has proven to be a powerful and general method to understand lncRNA localization. In this method, fluorescent antisense probes are hybridized to RNA targets in fixed cells and visualized using fluorescence microscopy. Shown on the *left* by FISH is the Xist RNA cloud coating the Xi after Xist induction. However, the resolution provided by this method is limited to relatively coarse observations. Depicted on the *right* is the same Xist induction time course assayed by RNA antisense purification (RAP) of Xist RNA using biotinylated antisense probes and sequencing of the bound X chromosome DNA. Such methods revealed that Xist exploits the 3D structure of the X chromosome to initially bind sites spatially proximal to the Xist locus and then spread to nearby active regions. (From Engreitz et al. 2013, adapted, with permission.)

ralen, enables the mapping of different interaction types, including protein-mediated RNA localization and direct nucleic acid hybridization (Engreitz et al. 2014).

Application of these methods to map Xist interactions with chromatin shed new light on how Xist spreads across the X chromosome during XCI in inducible Xist cell lines and female ESCs (Engreitz et al. 2013; Simon et al. 2013). These studies identified discrete and linearly distal sites on the Xi where the Xist RNA first localizes before spreading broadly across the X chromosome. Interestingly, the initial Xist localization sites lack enrichment for specific sequence motifs or chromatin modifications. Instead, comparison to

high-throughput chromatin conformation capture (Hi-C) interaction data revealed a striking relationship between the initial Xist binding sites and DNA regions that are in close 3D physical proximity to the XIC in the nucleus. This observation led to the model that Xist exploits 3D chromosome conformation, rather than specific high-affinity sequences, to identify its initial target sites on the Xi. In fact, determining the localization of Xist when it is expressed from a transgene located on a distinct region of the X chromosome showed that the RNA then localized at sites that were closest in 3D proximity to the transgene integration site—displaying an entirely distinct pattern of locali-



zation from that observed when Xist was expressed from its endogenous locus (Engreitz et al. 2013).

These genomic mapping tools were also essential for uncovering how Xist spreads from its site of transcription to other sites on the Xi. Specifically, Xist spreads from its initial target sites to more distal sites on the X chromosome. This spreading to actively transcribed regions on the X chromosome requires the A repeat region of Xist (Chaumeil et al. 2006; Engreitz et al. 2013; Chen et al. 2016), a region that is necessary for transcriptional silencing (Wutz et al. 2002). These studies revealed that Xist initially localizes to DNA regions that most frequently contact the Xist genomic locus in 3D proximity (Engreitz et al. 2013). At each of these sites, Xist interacts with the nuclear matrix protein SAF-A, which provides the physical link that tethers the Xist lncRNA to genomic DNA (McHugh et al. 2015). One of the most striking architectural changes associated with the induction of Xist is the repositioning of the entire Xi adjacent to the nuclear lamina. Recent proteomic studies have revealed that Xist directly interacts with lamin B receptor (LBR), and disruption of the Xist–LBR interaction leads to defective recruitment of the X to the lamina, as well as preventing Xist from spreading (McHugh et al. 2015; Minajigi et al. 2015). These results showed that Xist interacting with LBR leads to a change in chromosome architecture and enables Xist spreading from its site of transcription to other sites on the Xi (Engreitz et al. 2013; Chen et al. 2016).

Although these methods have provided critical insights for studying lncRNAs, there are still many important issues that need to be taken into account when interpreting the data. Specifically, capture of any RNA will enrich its transcribed DNA locus and accordingly, it is extremely challenging to discriminate between lncRNA interactions that occur in *cis* from those that simply reflect nascent transcription. In the case of Xist, this was addressed by using a doxycycline inducible promoter to perform a pulse-chase experiment and prevent nascent transcription of Xist before mapping its localization, ensuring that the DNA sites identified accurately represented the lncRNA targets (Engreitz et al. 2013). Given that many lncRNAs are thought to act in *cis*, future work is required to develop more generally applicable solutions that address this challenge directly.

4.3 How Localization Provides Insights into lncRNA Mechanisms

These same microscopy and sequencing approaches have been used to study the localization of other lncRNAs and derive key insights into their mechanisms.

For example, FISH experiments revealed that the imprinting lncRNAs Air (Nagano et al. 2008) and Kcnq1ot1 (Terranova et al. 2008) accumulate along the chromosome

near their sites of transcription only on the same allele, colocalizing with repressive chromatin modifiers and marks, suggesting that these RNAs may mediate repression of neighboring genes in *cis*. Conversely, FISH against the abundant highly conserved lncRNAs MALAT1 and NEAT1 revealed that they localize in many different nuclear sites corresponding to nuclear speckles and paraspeckles, respectively (Clemson et al. 2009; Mao et al. 2011). Sequencing studies showed that both Malat1 and Neat1 localize broadly to hundreds of transcribed DNA loci (Engreitz et al. 2014; West et al. 2014). In the case of Malat1, the lncRNA specifically associates with the nascent pre-mRNA at these sites and the interaction is rapidly abrogated on transcriptional inhibition (Engreitz et al. 2014; West et al. 2014). Finally, FIRRE was shown to accumulate over an ~5 Mbp region at its site of transcription on the X chromosome, and it also localizes to a number of seemingly distal autosomal sites. FISH on FIRRE showed that it concentrates in only one nuclear focus and that the *trans* targets are spatially organized within this nuclear compartment (Hacisuleyman et al. 2014). Despite these important insights derived from localization studies, it is still unclear what roles most of these lncRNAs play in organizing these precise locations within the nucleus (Engreitz et al. 2016b).

These examples highlight two strategies by which a lncRNA can localize in the nucleus—spatial proximity and affinity to targets. The balance between lncRNA localization through spatial proximity or DNA affinity may depend on the abundance of a lncRNA. For example, low-abundance lncRNAs may preferentially interact with regions that are in close proximity because they can attain high local concentration at these regions, whereas abundant lncRNAs may be able to diffuse through the nucleus to achieve high concentration at multiple target regions in *trans*. As an example, the HOTTIP lncRNA is expressed at low levels (about one copy per cell) and localizes in direct proximity to its transcription locus (Wang et al. 2011). In contrast, Malat1 is highly expressed (thousands of copies per cell) and can localize broadly to many different actively transcribed DNA regions throughout the nucleus (Engreitz et al. 2014; West et al. 2014). Although these lncRNAs represent examples of these respective localization strategies, other lncRNAs can integrate features of both strategies. For example, the roX2 lncRNA binds to specific DNA regions that are bound by the CLAMP protein, but does so preferentially at binding sites that are in close spatial proximity to its genomic locus (Soruco et al. 2013).

Although most well-studied lncRNAs accumulate in the nucleus, a number have been found in the cytoplasm, suggesting functions in posttranscriptional cellular processes. The most comprehensive lncRNA localization study to date used single-molecule FISH to examine the subcellular lo-

calization and abundance of 61 lncRNAs in three cell types, and showed that nearly half exist in the cytoplasm (Cabili et al. 2015). Although cytoplasmic functions of lncRNAs are not well understood, a number of studies have used the methods described here to begin to describe the diverse roles lncRNAs can play in regulating cellular processes outside the nucleus. These include blocking posttranslational modifications on signaling proteins, acting as decoys for proteins and miRNAs, and regulating mRNA stability and translation (Rinn and Chang 2012; Engreitz et al. 2016b; Kopp and Mendell 2018).

5 FUTURE DIRECTIONS: lncRNAs AND PHASE SEPARATION IN THE NUCLEUS

An emerging theme from studies of Xist, Malat1, Neat1, Firre, and other lncRNAs is their role in organizing nuclear architecture. lncRNA expression creates a high local concentration near its site of transcription in 3D space, scaffolding the assembly of chromatin and RNPs into a structural domain (Fig. 5). Such domains could serve to locally coordinate shared regulatory processes (e.g., nucleoli, Cajal bodies, nuclear speckles) (Shevtsov and Dunder

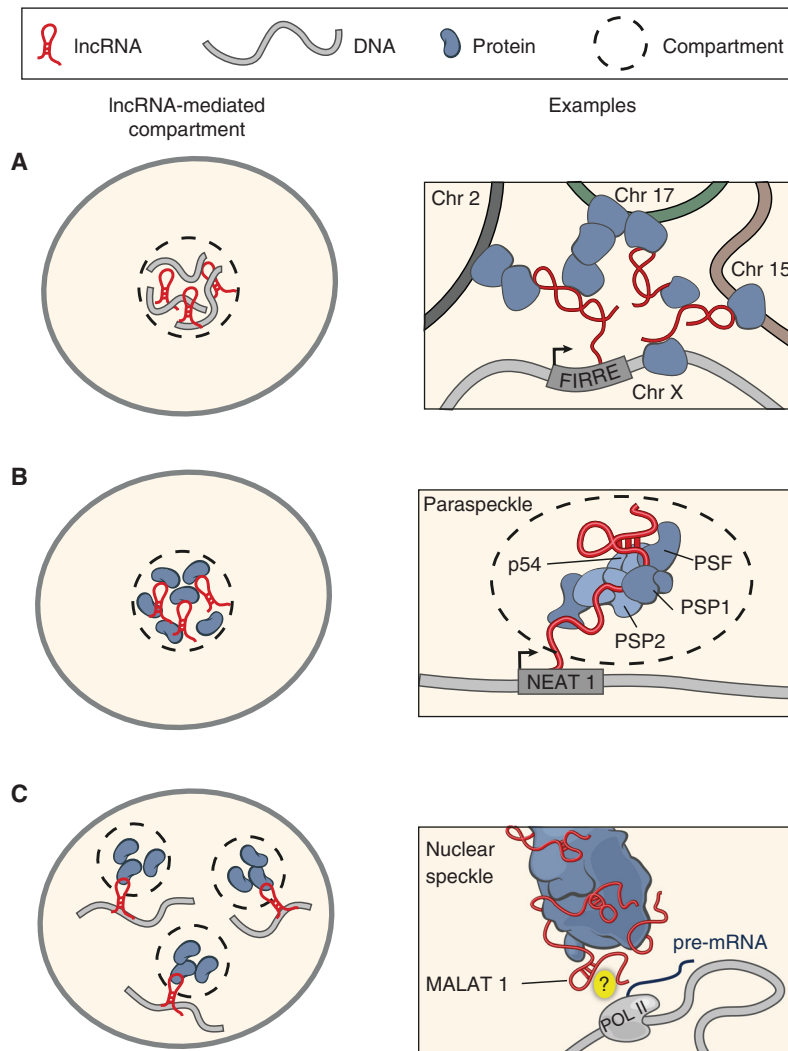


Figure 5. Examples of long noncoding RNAs (lncRNAs) forming dynamic spatial compartments in the nucleus by interacting with DNA, RNA, and protein. (A) *Firre* interacts with multiple functionally related loci on different chromosomes, brought together into a single nuclear territory for co-regulation. A high local concentration of *Firre* is required to form this structure. (B) *Neat1* expression nucleates paraspeckle formation in the nucleus, creating phase-separated domains that may also contain additional RNA species. It is unclear if the role of paraspeckles is to coordinate RNA biogenesis and processing, or to trap and sequester molecules away from the rest of the nucleus. (C) *Malat1* binds nascent pre-mRNAs and splicing factors in nuclear speckles and also interacts with hundreds of actively transcribed DNA loci, potentially integrating transcription and splicing within the same nuclear structure.



2011), bring together multiple chromosomal loci for common chromatin modifications within nuclear territories (e.g., Polycomb bodies and the inactive X) (Delest et al. 2012), or sequester RNA binding proteins and regulatory RNAs to titrate them away from other regions of the nucleus (e.g., paraspeckles) (Mao et al. 2011; Fox et al. 2018). In addition to nucleating functional domains within the nucleoplasm, lncRNAs can also tether these structures to chromatin through association with DNA-binding proteins such as SAF-A/hnRNP, and localize them to particular nuclear territories, as is the case with Xist interacting with LBR to associate the Xi with the nuclear lamina (Chen et al. 2016), coordinating higher levels of organization.

A model of liquid–liquid phase separation in the nucleoplasm has recently been proposed to explain the dynamic formation of membraneless nuclear compartments to coordinately regulate different aspects of gene expression (Hyman et al. 2014; Engreitz et al. 2016b; Hnisz et al. 2017). A central tenant of this model is that cooperative interactions between high local concentrations of nucleic acids and proteins—particularly those with intrinsically disordered domains, present in many RBPs—can lead to the formation of phase-separated bodies that compartmentalize biochemical functions in cells. High local concentrations of lncRNAs recruiting and concentrating proteins into lncRNA–protein structures could nucleate such events, thus dynamically organizing nuclear structures and functions into discrete domains (Fig. 5). In addition, lncRNAs may also act to create phase separated compartments within the nucleoplasm or cytoplasm. Such phase separated structures, however, are large and complex, containing dozens to thousands of distinct chromatin, RNA, and protein components occupying relatively large volumes in cells. Current methods such as microscopy, RNA purification, and Hi-C can be combined to study the individual components of nuclear bodies and territories, but are incapable of comprehensively characterizing how molecules simultaneously associate to function within an individual nucleus. A new method recently developed uses split-and-pool barcoding of cross-linked nucleic acids and proteins to define their associations in higher-order structures (Quinodoz et al. 2018). In the future, such methods will allow simultaneous mapping of multiple RNAs and DNA targets that co-occur within 3D structures in the nucleus. In addition, new developments in CRISPR-based live-cell imaging will make it possible to map large numbers of RNA and DNA structural dynamics as they occur in real time (Maass et al. 2018), a critical requirement for studying the role of RNA in phase separation, shaping genome organization, and gene regulation across time.

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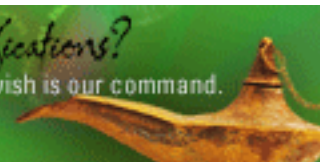


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